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MECHANICAL STRAIN INDUCES Cbfa1 AND TYPE X COLLAGEN EXPRESSION IN MANDIBULAR CONDYLE

Sze Van Lam, and A. Bakr M. Rabie

Biomedical and Tissue Engineering Research Group and Orthodontics, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, Hong Kong SAR, China

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1. ABSTRACT

Core binding factor a1 (Cbfa1) is a crucial transcriptional factor for chondrocyte maturation and osteoblast differentiation in the mandibular condyle. To quantitatively assess the amount of mRNA expression of Cbfa1 and type X collagen in response to mandibular advancement. 420, 35-day-old female Sprague-Dawley rats, were randomly divided into 20 experimental and 10 control groups corresponding to 10 time points. Experimental animals were advanced in either single or stepwise manner. Condylar cartilage was dissected under microscope and total RNA was extracted, Cbfa1 and type X collagen mRNA was quantified with real-time RT-PCR. Cbfa1 and type X collagen mRNA expression for all groups reached their peak on experimental day 21. During single advancement, Cbfa1 and type X collagen mRNA expression was consistently higher (3-fold and 2.8-fold respectively) than that of stepwise advancement (2-fold). The second advancement in the stepwise group resulted in both Cbfa1 and type X collagen level reaching another peak on day 51. Mandibular advancement promoted chondrocytes maturation and osteoblast differentiation by upregulating Cbfa1 expression. Stepwise advancement produced a higher level of Cbfa1 and type X collagen expression leading to bigger cartilage template onto which bone will form through endochondral ossification.

2. INTRODUCTION

The mechanotransduction pathway through which the cells receive and convert mechanical signals into tissue growth in the mandibular condyle is still being elucidated. Changing the biophysical environment of the temporomandibular joint by stretching the condylar attachment in response to moving the mandible forward offers an excellent model to study the mechanotransduction pathway (1). In response to such changes in the biophysical environment of the joint, the number of replicating mesenchymal cells increases (1, 2). Such an increase in the number of mesenchymal cells directly impacts the growth potential of the condyles. Resident mesenchymal cells in the proliferative layer give rise to chondroblasts and chondrocytes and proceed to form cartilage, the template onto which bone will form (3). Whereas mesenchymal cells that give rise to osteoblasts are present in the perivascular sites of the invading new blood vessels at the erosive zone in the condyle (4). Therefore, the higher the number of mesenchymal cells in a given site, the more the potential of bone growth at that site (5). Rabie and co-workers reported a close correlation between the number of mesenchymal cells and bone formation in the condyle and glenoid fossa (1, 6).

Naturally, one should ask what are the factors that regulate the number of mesenchymal cells in the

Table 1. Primers Used in This Paper with Their Orientation, Sequence, Melting Temperature (Tm), Length of Amplicon (bp) and Number of Cycles Used in the RT-PCR Protocol

Primer	Primer / Probe	Sequence	Tm (°C)	Amplification Product (bp)	Number of Cycles
Cbfa1	Forward Reverse	TTCTCCAACCCACGAATGCAC CAGGTACGTGTGGTAGTGAGT	60	108	45
Collagen X	Forward Reverse	TTCTGCTGCTAGTGTCCTTGACG GGGATGAAGTATTGTGTCCTGGG	60	115	45
GADPH	Forward Reverse	CATGTTCCAGTATGACTCTACCC AGCATCACCCCATTTGATGT	60	136	45

condyle? Several factors have been reported. First, genetic controls, the number of mesenchymal cells is genetically controlled (7, 8). Second, factors regulating mesenchymal cell proliferation (9) and differentiation (3). Third, the speed of maturation of chondrocytes to hypertrophic chondrocytes (10). This point is of great importance because the delay in maturation of chondrocytes allows mesenchymal cells to continue to replicate up to their full potential of 38 $\pm$ 4 cycles. PTHrP was found to be responsible for the delay of maturation of mesenchymal cells in the condyle (10). Whereas Core binding factor a1 (Cbfa1) was found to be responsible for the progression of chondrocytes into hypertrophic chondrocytes and thereby end further cellular proliferation (11). Fourth, the factors that regulate the differentiation of mesenchymal cells into osteoblasts in the erosive zone of the condyle. Recently, it was reported that Cbfa1 is a factor that affects the osteoblasts differentiation and chondrocytes maturation and couples chondrocytes maturation and endochondral ossification during natural growth of the mandible (11). Such an orchestrated influence of PTHrP and Cbfa1 is critical to condylar growth because it regulates the speed of chondrocyte maturation and subsequently affects cellular proliferation. It would be of great interest to determine if condylar growth induced by forward mandibular positioning would solicit such a response.

Therefore the purposes of this study are to quantitatively assess the mRNA expression of Cbfa1 and type X collagen during natural growth and in response to single-step and stepwise mandibular advancement; and to correlate Cbfa1 expression to the pattern of expression of type X collagen. Hoping to understand the tissue response to a treatment modality commonly used in the clinic.

3. MATERIALS AND METHODS

Animal experiment was approved by the committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

3.1. Experimental animals

Four hundred and twenty 35 days old female Sprague-Dawley rats were allocated into 10 control and 20 experimental groups. 10 groups of experimental rats were randomly selected to wear the single-step bite-jumping appliance and another 10 groups to wear the stepwise bite-jumping appliance which positioned the mandible forward in a step-by-step manner. The single-step bite-jumping appliance positioned the mandible forward giving a 4mm mandibular advancement in the sagittal plane (12, 13). Stepwise appliance had an initial 2mm anterior advancement and veneers were placed on day 30 added for another 2mm advancement. The appliances were worn 24 hours producing a continuous forward and downward positioning of the mandible. Animals in each experimental and corresponding control group were sacrificed on days 3, 7, 14, 21, 30, 33, 37, 44, 51 and 60 by overdose intraperitooneal injection of pentobarbital sodium.

3.2. RNA extraction

Immediately after death, the mandibular condyles were collected in RNAlater^{TM} (Qiagen) and the mandibular condylar cartilage was dissected under dissecting microscope. The condylar cartilage was homogenized with Mikro-dismembrator U (B. Braun Biotech International). Total RNA was extracted from the condylar cartilage by means of the RNeasy Fibrous Tissue Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Quality of the extracted RNA was assessed by Bioanalyser 2100 (Agilent technologies).

3.3. Reverse transcription and real-time polymerized chain reaction

Reverse transcription (RT) was carried out on a PRC Thermal Cycler (Takara) with the SuperScript First-Strand Synthesis System using the manufacturer's protocol (Invitrogen). Real-time PCRs were carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The cycle profile was as follows: a hold at 94°C for 2 min for denaturation, 45 cycles of amplification at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. The fluorescence signals were measured and plotted against the temperature. The transcript levels of Cbfa1 and type X collagen in different samples were analyzed using the iCycler iQ^{TM} Real-Time PCR Detection System Software (Bio-Rad Laboratories, Hercules). To correct for differences in both quality and quantity between samples, data were normalized using the ratio of the measured gene concentration to that of glyceraldhyde-triphosphate dehydrogenase (GAPDH), which was assessed in a different reaction in the same experimental round.

After the final cycle of real-time PCR, specificity of the reaction was checked by melting curve analysis as follows: a hold at 95°C for 60 sec, a hold at 55°C for 60 sec, and a slow ramp from 55°C for 10 sec then 0.5°C increase every 10 sec up to a final temperature of 98°C (Figure 1). Moreover, to further confirm the specificity of PCR, the products were subjected to electrophoresis on a 1.0% (w/v) agarose gel (Figure 2), bands of expected size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen), and then sequenced directly by the same forward or reverse primers used in the original PCR amplification. The DNA sequencing revealed that the RT-PCR amplified Cbfa1, type X collagen and GAPDH

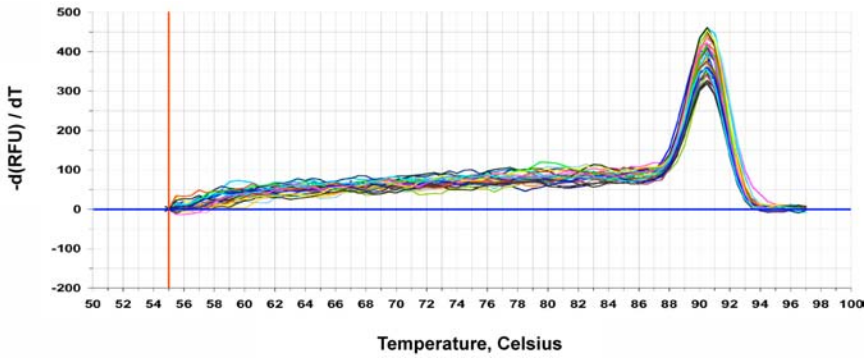

Figure 1. Melting curve analysis in real-time PCR. A sharp peak showing specific Cbfa1 amplicon (melting temperature of 90.5^{o}C) was generated.

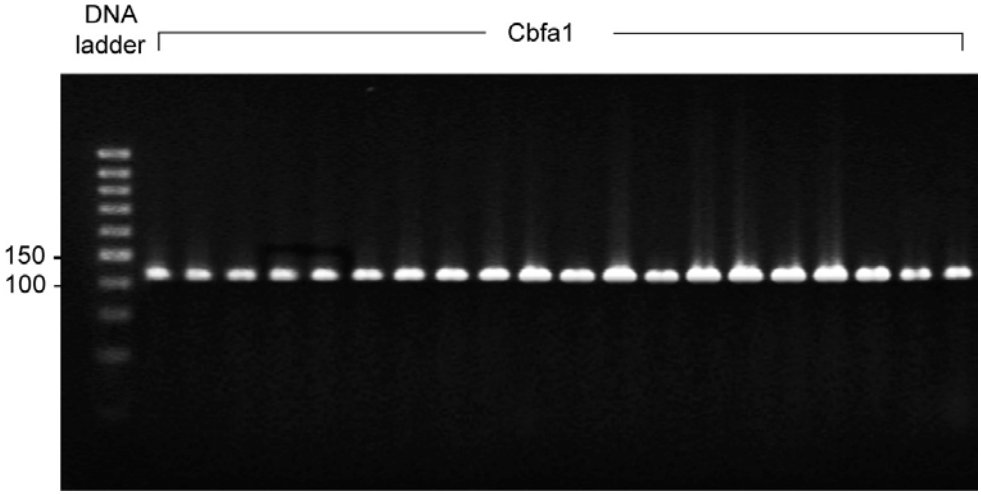

Figure 2. Analysis of final PCR products using gel electrophoresis with 50bp DNA ladder. Cbfa1 with amplicon product size of 108bp showing high specificity.

products were consistent with NCBI gene bank (GI: 34874772, 1086927 and 37590766 respectively). The identities ranged from 95 to 100%. Thus, showing a high specificity of the reactions.

3.4. Primer sequence

Oligonucleotide primers sets specific for Cbfa1, type X collagen and GAPDH for quantitative real-time PCR (Table 1) were obtained from the PrimerBank database (14).

3.5. Statisticical Analysis

The data were processed with SPSS (version 11.0) for one-way anova with bonferroni adjustment to compare the mean differences of mRNA expression among the control group and the two experimental groups at each time point.

4. RESULTS

4.1. Cbfa1 expression in mandibular condylar cartilage

During natural growth, the level of Cbfa1 messenger RNA progressively increased between 1 and 21 experimental days, reaching a peak at experimental day 21 after which the expression declined gradually with age (Figure 3).

Forward mandibular positioning led to a significant increase of Cbfa1 expression on day 14 for both experimental groups (3-fold increase for single advancement and 2-fold increase for stepwise group) and reached maximum level on day 21.

In the single advancement group, Cbfa1 expression was consistently higher when compared to that of the stepwise group over day 7 to 30 as well as the normal growth. Cbfa1 expression decreased rapidly from experimental day 21 to levels equal to that expressed in the untreated controls. In the stepwise advancement group, Cbfa1 expression showed a similar temporal pattern to the single advancement group from day 3 to day 30. Upon second mandibular advancement on experimental day 30, a significant increase of Cbfa1 expression was detected on day 44 and day 51, registering a fold change of 3 and 2.8

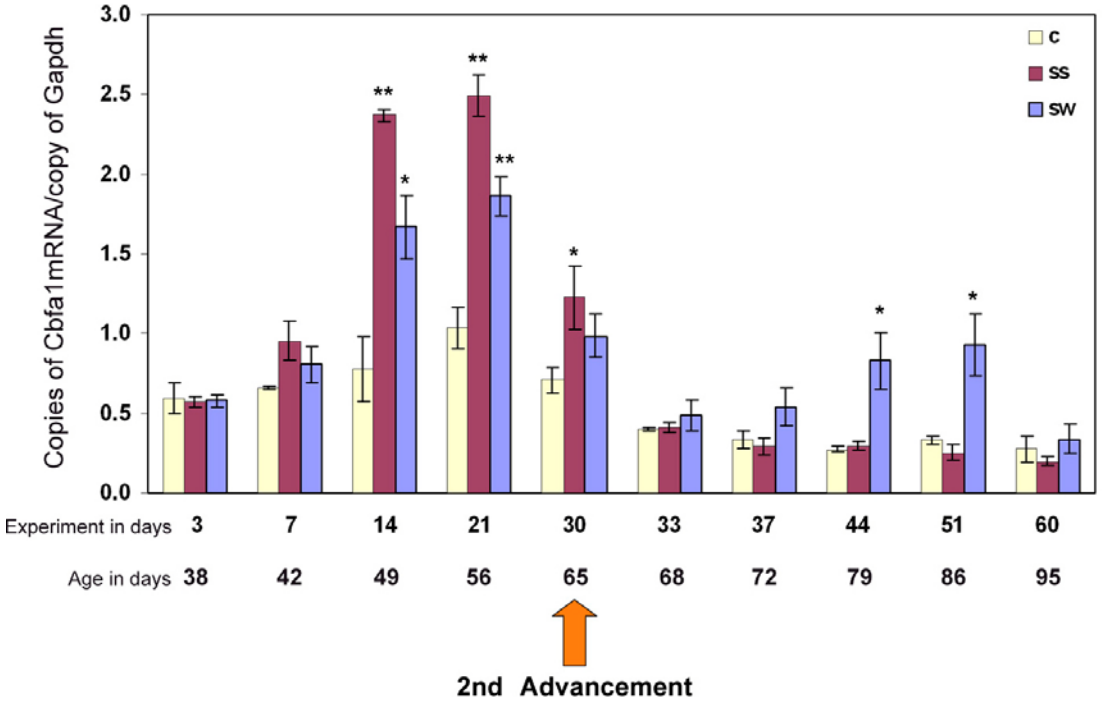

Figure 3. Quantitative analysis of Cbfa1 mRNA expression in condylar cartilage with real-time RT-PCR using SYBR Green during growth (control) and mandibular forward positioning in single-step (ss) and stepwise (sw) advancement (*P<0.05, **P<0.01).

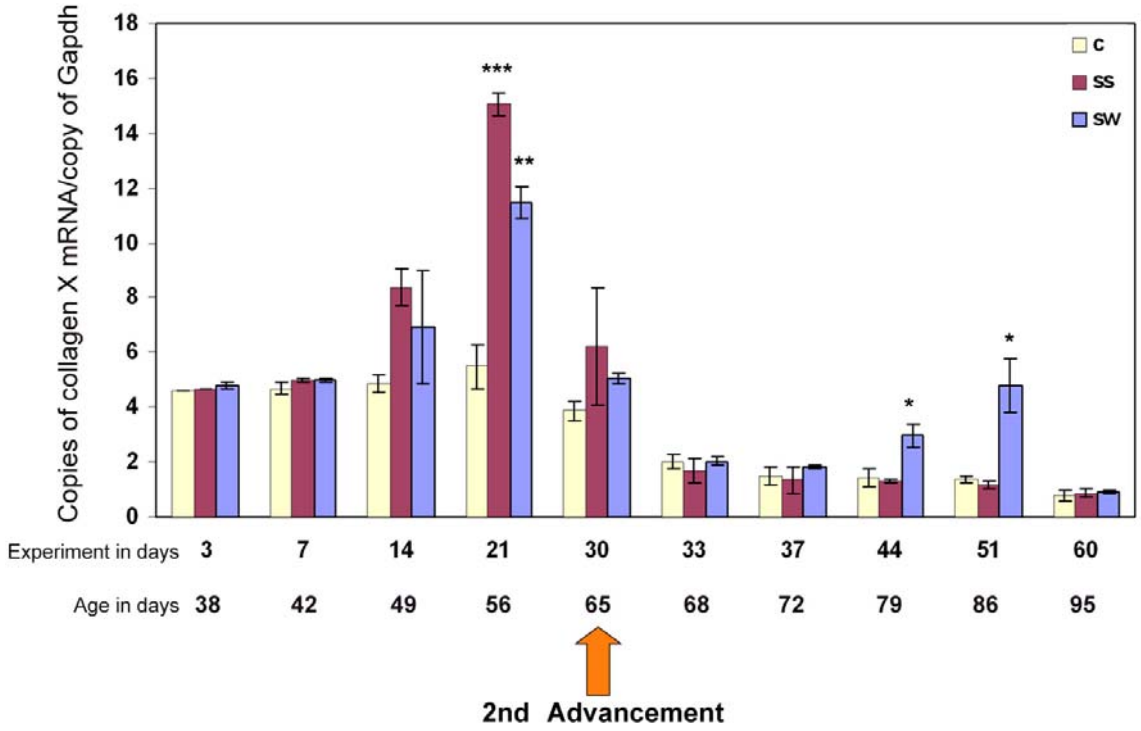

Figure 4. Quantitative assessment of Type X collagen mRNA expression in condylar cartilage with real-time RT-PCR using SYBR Green during growth (c) and mandibular forward positioning in single-step (ss) and stepwise (sw) advancement (*P<0.05, **P<0.01, ***P<0.001).

respectively when compared to both the single advancement as well as the untreated.

4.2. Type X collagen expression in mandibular condylar cartilage

During natural growth, the amount of type X collagen messenger RNA decreased gradually as the rat aged (Figure 4).

In the single step advancement group, type X collagen expression remained low on experimental day 7. On day 14, an increase in the expression level was detected. However, the difference was statistically insignificant. This is followed by a significant increase to the highest level on experimental day 21, corresponding to a fold change of 2.8. The expression decreased markedly after the peak and dropped below the control level.

In the stepwise advancement group, a similar temporal pattern was observed during the first 30 days of mandibular advancement where the condylar cartilage in the single step group consistently expressed a higher level of type X collagen mRNA. The second mandibular advancement on day 30 led a significant increase of type X collagen expression on day 44 and day 51. This gave a fold change of 2 and 3.5 respectively when compared with the corresponding controls.

5. DISCUSSION

Results of the current study clearly demonstrated that bouts of loading the mandibular condyles through mandibular advancement in a stepwise manner trigger re-activation of cartilage synthesis and bone formation under the tight control of Cbfa1. The pattern of expression of Cbfa1 during forward mandibualr positioning was similar to that identified during natural growth of the condyle. Indicating that condylar growth induced by forward mandibular positioning mirrors natural growth. However, the levels of expression of Cbfa1 mRNA were significantly higher in the advancement group when compared to natural growth (Fig 3). The higher levels of Cbfa1 expression is indicative of the entry of chondrocytes into the hypertrophic chondrocytes maturation stage that expresses type X collagen and marks the beginning of endochondral ossification. This is why in the present study the maximum level of expression of Cbfa1 coincided with the maximum level of expression of type X collagen (Figs 3, 4). Type X collagen, the major component of the hypertrophic collagen matrix that is replaced by bone, marks the onset of endochondral ossification in the condyle (4). This is why the highest level of expression of Cbfa1 preceded the maximum increase in bone formation in the condyle in a matched sample (15). The importance of Cbfa1 in chondrocyte maturation was clearly demonstrated by Otto and co-workers (16) where maturation arrest of chondrocytes in Cbfa1 deficient mice was noted. Furthermore, Cbfa1 is essential for the differentiation of mesenchymal cells into osteoblasts in the erosive zone and subsequently it is important for bone growth in the condyle (11). The critical role of Cbfa1 in bone formation was clearly demonstrated in Cbfa1 deficient embryos where no calcification occurred in the skull, mandible, humerus or femur while wild type exhibited extensive calcification of all the skeletons (16). The absence of calcification of the mandible in Cbfa1 deficient mice and its over expression during condylar growth caused by mandibular advancement points to the role played by Cbfa1 in regulating post-natal growth of the condyle during growth modifications of the mandible.

Another interesting finding in this study was the results of the stepwise advancement compared to single advancement (Figs 3, 4). The second advancement resulted in reactivating osteoblasts differentiation leading to more new bone formation indicated by the significant increase in Cbfa1 mRNA expression and type X collagen mRNA expression when compared to natural growth (Figs 3, 4). The current results support and explain earlier clinical findings where stepwise advancement of the mandible resulted in a better skeletal treatment effect on mandibular growth when compared to single step advancement (17). It also supports earlier experimental work by Petrovic et al. (18) where periodic forward repositioning of the mandible appeared to cause an increase in the rate and amount of condylar cartilage growth. Obviously, the significant increase in Cbfa1 expression could have contributed to the apparent increase in the rate as it speeds up the maturation of the chondrocytes. Furthermore, Forwood and Turner (19) reported that bouts of loading the joint in long bones resulted in recruitment of osteoblasts in a quantum fashion. It is possible that in the mandibular condyle, the stepwise advancement through the regulatory effect of Cbfa1, could have recuited the osteoblasts differentiation and led to more bone formation. This was confirmed in an earlier report where mandibular advancement in a stepwise manner resulted in significantly more new bone formation in response to the second advancement when compared to both single advancement group and matched untreated controls (15). This points out the role played by Cbfa1 in osteoblast differentiation in the erosive zone in the condyle.

6. CONCLUSION

Cbfa1 is an important factor that regulates post-natal growth of the condyle induced by forward mandibular positioning. It controls the maturation of chondrocytes into hypertrophic chondrocytes and regulates the differentiation of mesenchymal cells into osteoblasts that proceed to make bone in the condyle.

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Send Correspondence to: Professor A.B.M. Rabie, Orthodontics, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, Hong Kong SAR, China. Tel: 852-28590260, Fax: 852-25593803, E-mail:rabie@hkusua.hku.hk

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